Serum and Mucosal Immune Responses to an Inactivated Influenza Virus Vaccine Induced by Epidermal Powder Immunization

Dexiang Chen, Sangeeta B. Periwal, Katherine Larrivee, Cindy Zuleger, Cherie A. Erickson, Ryan L. Endres and Lendon G. Payne

Serum and Mucosal Immune Responses to an Inactivated Influenza Virus Vaccine Induced by Epidermal Powder Immunization

DEXIANG CHEN,* SANGEETA B. PERIWAL,† KATHERINE LARRIVEE, CINDY ZULEGER, CHERIE A. ERICKSON, RYAN L. ENDRES, AND LENDON G. PAYNE

PowderJect Vaccines, Inc., Madison, Wisconsin 53711

Received 12 February 2001/Accepted 29 May 2001

Both circulating and mucosal antibodies are considered important for protection against infection by influenza virus in humans and animals. However, current inactivated vaccines administered by intramuscular injection using a syringe and needle elicit primarily circulating antibodies. In this study, we report that epidermal powder immunization (EPI) via a unique powder delivery system elicits both serum and mucosal antibodies to an inactivated influenza virus vaccine. Serum antibody responses to influenza vaccine following EPI were enhanced by codelivery of cholera toxin (CT), a synthetic oligodeoxynucleotide containing immunostimulatory CpG motifs (CpG DNA), or the combination of these two adjuvants. In addition, secretory immunoglobulin A (sIgA) antibodies were detected in the saliva and mucosal lavages of the small intestine, trachea, and vaginal tract, although the titers were much lower than the IgG titers. The local origin of the sIgA antibodies was further shown by measuring antibodies released from cultured tracheal and small intestinal fragments and by detecting antigen-specific IgA-secreting cells in the lamina propria using ELISPOT assays. EPI with a single dose of influenza vaccine containing CT or CT and CpG DNA conferred complete protection against lethal challenges with an influenza virus isolated 30 years ago, whereas a prime and boost immunizations were required for protection in the absence of an adjuvant. The ability to elicit augmented circulating antibody and mucosal antibody responses makes EPI a promising alternative to needle injection for administering vaccines against influenza and other diseases.

Influenza, with recurrent worldwide epidemics, presents a major health problem. It affects hundreds of millions of people every year, with high morbidity in people of all ages and high mortality in high-risk populations (19, 43). In the United States alone, infections with influenza virus result in approximately 20,000 deaths and 100,000 hospitalizations each year (7). Influenza virus, the causative agent of influenza, is spread from person to person by aerosol. Infections are normally restricted to the upper respiratory tract unless the subject has a compromised immune system, e.g., the elderly or those with underlying diseases. Annual vaccination remains the most effective means of controlling influenza.

Immunization with live attenuated influenza viruses results in the production of circulating immunoglobulin G (IgG) antibodies and secretory IgA (sIgA) antibodies in the nasal secretion to a major surface glycoprotein, hemagglutinin (HA). Both circulating IgG and sIgA antibodies have been correlated with protection (3, 5, 10). The current influenza vaccine consists of three split influenza viruses, which may vary from year to year, and is administered by intramuscular (i.m.) injection using a syringe and needle (7). A fourfold rise in serum antibodies to HA measured using the hemagglutination inhibition (HI) assay is indicative of seroconversion. Unlike natural infections, i.m. administration of inactivated vaccine, although eliciting serum antibodies, does not usually induce mucosal immune responses, which are considered important for protecting the upper respiratory tract (4, 11, 15, 31). The protection rate by the current vaccine varies and is particularly low for the elderly population (6, 12, 21, 33). This vaccine is effective in reducing the number of severe cases of influenza and complications involving the lower respiratory tract but is less effective in reducing the rate of infection in the upper respiratory tract. Protection of the lower respiratory tract is believed to be through the circulating antibodies that transude into the lungs. The transuded antibody may not reach a protective concentration in the upper respiratory tract.

Mucosal immunity acts as the first line of host defense by blocking influenza virus from infecting the upper respiratory tract and spreading to the lower respiratory tract and deeper tissues (11). Therefore, an efficacious influenza vaccine should induce sIgA antibodies in the respiratory tract. Introduction of antigen via a nonmucosal route, e.g., i.m., subcutaneously, or intravenously, has been shown to be ineffective in turning on the mucosal immune system because of the compartmentalization of the immune system. Direct antigenic challenge across the mucosal surface is typically required to induce a mucosal immune response (22, 24, 28, 34). Mucosal immunization with the current split influenza vaccine, although attractive, faces significant challenges due to the lack of a safe and efficient delivery system and/or adjuvant. Consequently, mucosal immunization often requires a relatively large vaccine dose and, sometimes, repeated administrations, which may result in an increased cost and delay in achieving protective immunity (18, 20, 30, 32). Therefore, there is a need for new vaccines and vaccination technologies that can effectively induce both systemic and mucosal immunity.
We have previously reported that epidermal powder immunization (EPI) with influenza vaccine elicited a protective immune response in mice (9). Commonly used adjuvants including alum salts and CpG DNA can be used with EPI to further enhance antibody responses and modulate T-helper-cell activity (8). In this study, we present evidence that EPI of mice with influenza vaccine with adjuvants induces antigen-specific circulating antibodies, mucosal antibodies, and enhanced protection.

**MATERIALS AND METHODS**

**Vaccines and powder formulations.** Trivalent influenza vaccine (1998–1999) was supplied by Parkedale Pharmaceuticals (Rochester Hill, Mich.). It consisted of three split viruses: A/Sydney/5/97-like (H3N2) (A/Sydney), A/Beijing/262/95-like (H1N1) (A/Beijing), and B/Harbin/7/94-like (B/Harbin). A monovalent component was used in some studies. The A/PR/8/34 (H1N1) (PR8) influenza virus was purchased from Charles River Spafas (New Franklin, Conn.) and inactivated with formalin (1:4,000 [vol/vol]) for 48 h at 4°C. The phosphorothioate-modified CpG DNA oligonucleotide (ATCGACTCTC GAGCGTTCTC) (23) was synthesized at Midland Certified Reagent Company (Midland, Tex.). Cholera toxin (CT) was purchased from Sigma Chemicals (St. Louis, Mo.).

Vaccine powder formulations were prepared by an air-drying process (9). Appropriate amounts of vaccine, adjuvant, and trehalose solution (100 mg/ml in deionized water) were combined, incubated overnight at 4°C with shaking, and plated in a glass petri dish. The mixture was dried overnight in a desiccator (Nalgene, Rochester, N.Y.) purged with N2 gas. The dried solid was collected, ground with a pestle and mortar, and sieved using 3-μm-diameter stainless steel sieves. Dry powders of 20- to 53-μm-diameter fraction were loaded into a trilaminate cassette and used to immunize mice. Each administration delivered 1 mg of powder containing influenza vaccine alone or with either CpG DNA and/or CT.

**Mice and vaccination.** A previously described powder delivery system was used for this study (9). Female BALB/c mice (6 weeks old at the beginning of the study) from Harlan Sprague Dawley (Indianapolis, Ind.) were used for the study. Mice were fully anesthetized by intraperitoneal injection of ketamine and xylazine and their abdominal skin was clipped prior to powder injection. To perform EPI with powder formulations, the Powderject delivery device was placed against the shaved abdominal skin of animals and actuated by releasing compressed helium from the gas cylinder (9). Blood was collected via retro-orbital bleeding under anesthesia prior to each vaccination and 2 weeks postboost.

The subcutaneous (s.c.) injection was performed by injecting 0.25 ml of vaccine into the scuff of the neck using a 26-1/2-gauge needle.

**Collection of mucosal secretions.** Mucosal samples were collected using previously described methods (38), with modifications. Briefly, anesthetized mice were injected intraperitoneally with 0.1 mg of pilocarpine (Sigma). Secrected saliva was collected using a Pasteur pipette. Contents of the small intestine were collected and extracted with 1 ml of saline. Bronchial-alveolar lavage fluids were obtained by washing with 1 ml of saline. Vaginal washes were collected by washing with 75 μl of saline. All mucosal samples were clarified by centrifugation, and sodium azide and 50 μl of fetal calf serum were added as preservative and substrate for proteases. All samples were frozen at −80°C until assayed for antibody response.

**Mouse challenge.** A mouse-adapted A/Al/88/H1N2 (A/Alcihi) influenza virus was obtained from Yoshi Kawaoka (University of Wisconsin). The mouse-adapted Aichi virus was prepared by culturing in 10-day-old embryonated chicken eggs and purified using a previously described method (9). The 50% lethal dose (LD50) was experimentally determined to be 103 PFU. Mice were fully anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine and then intranasally instilled with 103 PFU (10 LD50) of the mouse-adapted Aichi virus in 50 μl of phosphate-buffered saline (PBS). Mortality and changes in body weight were monitored daily for 14 days and on day 21.

**ELISA.** The antibody titers to influenza vaccine were determined using a modified enzyme-linked immunosorbent assay (ELISA) (9). Formalin-inactivated influenza viruses (PR8s and A/Alcihi) or split influenza viruses (trivalent or monovalent) were used as detection antigens. Briefly, a 96-well Costar medium binding plate (Fisher Scientific Products, Pittsburgh, Pa.) was coated with 1 μg of influenza virus antigen per well in 30 mM PBS (pH 7.4) overnight at 4°C. Plates were washed and then incubated with test samples diluted in PBS containing 5% dry milk for 1.5 to 2 h at room temperature, followed by three washes and 1-h incubation with biotin-labeled goat anti-mouse IgG. Following three additional washes, plates were incubated with a streptavidin-horseradish peroxidase conjugate (Southern Biotechnologies) for 1 h at room temperature. Finally, plates were washed and developed for 15 min with the substrate 3,3′,5,5′-tetramethylbenzidine (Bio-Rad Laboratories, Malvile, N.Y.). The reaction was stopped by the addition of 1 N sulfuric acid. The plates were read at a 450-nm wavelength using an Emax plate reader ( Molecular Devices, Sunnyvale, Calif.). The endpoint titers of the sera were determined by four-parameter analysis using the Softmax Pro 4.1 program (Molecular Devices) and were defined as the reciprocals of the highest serum dilutions with an optical density reading above the background by 0.1. A reference serum with a predetermined titer was used on every plate to calibrate the titers and adjust assay-to-assay and plate-to-plate variation.

**Mucosal tissue fragment culture.** Mucosal tissues were collected from the immunized mice and used in the fragment culture assay to assess mucosal antibody production (38). Briefly, small pieces of trachea and small intestines were collected and washed extensively with calcium- and magnesium-free Hank’s balanced salt solution (GIBCO-BRL, Grand Island, N.Y.) containing 0.1% gentamicin. The tissue fragments were finally washed with complete RPMI and cultured in 24-well flat-bottom tissue culture plates for 7 days under 90% O2 and 10% CO2 at 37°C. The culture medium was Kennett’s H-Y medium (IRH Biosciences, Zelenza, Kans.) containing 10% fetal calf serum, 1% l-glutamine, 0.01% gentamicin, and 1% antibiotic-antimycotic solution. Influenza virus-specific antibodies in the tissue culture supernatant were assayed using an ELISA. The ELISPOT assay. IgA ELISPOT assays were performed using single-cell suspensions prepared from the lamina propria of the immunized mice as previously described (29). Cells were washed extensively with calcium- and magnesium-free Hank’s balanced salt solution and then resuspended in an RPMI 1640 medium containing 10% fetal calf serum, 1% l-glutamine, and 1% antibiotic-antimycotic solution.

ELISPOT assays were done in 96-well Immulon II plates (Millepore, Bedford, Mass.). Plates were coated with 10 μg of formalin-inactivated PR8 virus/ml in 0.1 M bicarbonate buffer, pH 9.5. Plates were blocked with 10% RPMI for 1 h at room temperature. After washing, 100 μl of lymphocyte suspensions from mucosal tissues at various concentrations was placed in each well and plates were incubated overnight at 37°C in 5% CO2. Plates were then washed five times with PBS. Then 100 μl of the appropriately diluted goat anti-mouse IgA–alkaline phosphate conjugate (Southern Biotechnologies) in PBS was added to each well, and the plates were incubated at room temperature for 4 h. Plates were again washed five times with PBS-0.05% Tween 80. Plates were developed using an alkaline phosphate substrate kit (Bio-Rad). Spots were quantified by counting spots under an Olympus dissecting microscope (Leeds Precision Instruments, Inc., Minneapolis, Minn.).

**IL-4 and IFN-γ ELISPOT assays.** Previously described ELISPOT assays were used to determine the cytokine responses (26). Briefly, 96-well Immulon II plates (Millepore) were coated with 1 μg of purified rat anti-mouse interleukin-4 (IL-4) monoclonal antibody (BV4-1D11; Pharmingen, San Diego, Calif.) or purified rat anti-mouse gamma interferon (IFN-γ) monoclonal antibody (R4-6A2; Pharmingen) well overnight at 4°C. Plates were blocked, washed with complete RPMI, and seeded with 100 μl of lymphocyte suspensions in twofold serial dilutions. Cells were stimulated with inactivated influenza virus at a final concentration of 5 μg/ml overnight in a 37°C humidified incubator with 5% CO2. One hundred microliters (4 μg/ml) of biotinylated rat anti-mouse IFN-γ (XMGl2; Pharmingen) or biotinylated rat anti-mouse IL-4 (2.5 μg/ml) (BV6D2-2462; Pharmingen) antibody was added the following day and incubated for 4 h at room temperature. Streptavidin–alkaline phosphatase conjugate (Mabtech, Nacka, Sweden) was added, and the plates were incubated at room temperature for 2 h. Plates were developed using an alkaline phosphatase substrate kit (Bio-Rad). The reaction was stopped by the addition of distilled water.

IL-4- or IFN-γ-secreting cells were quantified by counting spots under an Olympus dissecting microscope (Leeds Precision Instruments).

**Statistical analysis.** Data from ELISA and ELISPOT were analyzed using the Student's t test (JMP; SAS Institute Inc., Cary, N.C.). Some data were transformed logarithmically prior to statistical analysis.

**RESULTS**

**Serum IgG antibodies.** The ability of EPI to vaccinate mice (eight animals per group) was examined by administering one of the following powder formulations: 5 μg of PR8 influenza vaccine alone, PR8 coformulated with either (i) 5 μg of CT, (ii) 10 μg of CpG DNA, or (iii) 5 μg of CT and 10 μg of CpG...
DNA. A control group was given 5 μg of PR8 alone s.c. A booster immunization was administered on day 28. Blood samples were collected on day 0 and 14 days post-booster immunization, and antibody responses to the PR8 virus were measured using an ELISA.

All powder formulations elicited antigen-specific IgG antibody responses in serum (Fig. 1A). Compared to the titer elicited by PR8 alone, the titers were 5-fold higher for the CpG DNA formulation (P < 0.01), 30-fold higher for the CT formulation (P < 0.01), and 50-fold higher for the Ct formulation (P < 0.01). The CT formulation elicited a significantly higher IgG response than the CpG DNA formulation (P < 0.01). The control mice that were immunized s.c. had titers of less than 200. HI titers (C) were determined using pooled sera (n = 8).

The adjuvant activity of the powder formulations was differentiated when the IgG subclass titers were compared (Fig. 1B). PR8 virus alone administered by EPI elicited primarily IgG1 antibodies; the IgG2a or IgG2b antibody titers were much lower than the IgG1 titer. Although all IgG subclass titers increased with the use of CT, IgG1 antibodies remained the highest. IgG2a and IgG2b titers were equivalent to or higher than the IgG1 titer when CpG DNA was used alone or in combination with CT. The s.c.-immunized animals had considerably lower IgG subclass titers than mice receiving the EPI. The IgG1 antibodies were the predominant subclass in the control animals, with IgG2a and IgG2b antibodies being barely detectable.

The IgG1 titers measured in the ELISA correlated well with the HI titer (Fig. 1C). The HI titers were highest (>1,280) for the CT and the CT and CpG adjuvant formulations. EPI with PR8 alone elicited a fourfold higher titer than the s.c. immunization.

There were no significant PR8-specific IgA titers in any of the sera that could be detected by the ELISA assay. IgA antibodies may not be detectable when high IgG titers are present.

**IgG and IgA antibodies in mucosal secretions.** PR8-specific antibodies in the mucosal secretions of the animals in the

FIG. 1. Serum antibody responses. Mice were vaccinated on days 0 and 28 by EPI with PR8 alone or PR8 formulated with CpG DNA, CT, or CpG DNA plus CT. Control mice were immunized s.c. with 5 μg of PR8 alone. Blood samples were collected on day 42, and total IgG (A) and IgG subclass (B) antibody titers in serum were determined using an ELISA. Each data point represents the total IgG titer from eight mice, with geometric means indicated by bars. Preimmunization sera had titers of less than 200. HI titers (C) were determined using pooled sera (n = 8).
above study were also determined. On days 42 and 49, four animals from each group were sacrificed. Saliva, nasal wash, vaginal wash, and intestinal mucosal samples were collected and the PR8-specific IgG and IgA antibodies were measured using ELISA.

PR8-specific IgG antibodies were detected in the intestinal lavage (Fig. 2A), saliva (Fig. 2C), and vaginal washes (Fig. 2E) of mice immunized with the various powder formulations. The lowest IgG level was seen in mice immunized with PR8 alone and the highest IgG level was seen in mice immunized with PR8 plus the CT adjuvant or the CT and CpG DNA adjuvant combination. IgG antibodies in the nasal washes were not determined because of the sample shortage. The s.c.-immunized mice had undetectable or very low IgG antibody levels in the mucosal washes.

PR8-specific IgA antibodies were detected in the intestinal lavage (Fig. 2B), saliva (Fig. 2D), vaginal washes (Fig. 2F), and nasal wash fluids (Fig. 2G) from mice immunized with the various powder formulations. In the absence of adjuvants, powdered PR8 virus alone elicited low but detectable antigen-specific IgA antibodies in small intestines and nasal mucosa. IgA antibodies were consistently detected in all mucosal wash fluids when the CpG DNA or CT adjuvant was included in the vaccine. The highest IgA antibody levels were consistently seen in all mucosal wash fluids from mice immunized with PR8 virus plus the CpG DNA and CT adjuvant combination. Unlike the results achieved by EPI, there were no detectable PR8-specific IgA antibodies significantly above the background level in any of the mucosal wash fluids from the s.c.-immunized mice.

It should be noted that PR8-specific IgG titers were higher than IgA titers in all mucosal secretions.

**IgG and IgA antibodies from mucosal fragment culture.** In vitro tissue fragment cultures were examined to determine if the PR8-specific IgG and IgA antibodies observed in the mucosal washes originated from the mucosal tissue. When animals were sacrificed on days 42 and 49, tracheas and small intestines were collected, pooled by group from four mice each time, and cultured for 7 days in a modular incubator chamber.

The average IgG antibody levels in the culture supernatant, as determined in an ELISA, from two assays are shown in Fig. 3A. The levels of PR8-specific IgG antibodies are remarkably high for the powder formulations when compared to the s.c. control. EPIs with CpG DNA or CT adjuvant separately or in combination have elicited consistently increased PR8-specific IgG antibody levels when compared to the formulation without an adjuvant.

Although PR8-specific IgG is the dominant antibody in the supernatants of cultured mucosal tissue fragments, IgA antibodies were also detected (Fig. 3B). Antigen-specific IgA antibodies were detected in the cultured small intestine fragments for the powdered formulation with PR8 alone. PR8-specific IgA antibodies from cultured intestinal and tracheal fragments were consistently detected when CpG DNA or CT adjuvant was used in the powder formulation. However, the use of the adjuvant combination in the powder formulation led to the highest IgA titer towards PR8 virus. There were no appreciable IgA antibodies above the background of the assay in cultured mucosal fragments from the s.c.-immunized mice or naïve mice. These results suggested the presence of PR8-specific IgA-secreting plasma cells in the mucosal tissues at the time of sample collection in mice immunized by EPI but not s.c.

**Antigen-specific IgA-secreting cells in mucosal tissue.** A new experiment was performed to enumerate PR8-specific IgA-secreting cells in mucosal tissues of mice receiving EPI. In this study, two groups of mice (n = 8) were immunized by EPI on days 0 and 28 with 5 µg of PR8 formulated with 10 µg of CpG DNA and 5 µg of CT. The vaccination sites for one group were covered immediately after immunization with a piece of parafilm (15 mm in diameter), and the films were taped to the animals for 24 h. Covering the vaccination sites prevented oral uptake of antigens through animals licking the treatment sites of cage mates. Control mice were immunized by s.c. injection using the same vaccine and adjuvant in PBS. Two weeks postbooster immunization, lamina propria were collected and pooled by group. Single-cell suspensions were prepared and PR8-specific IgA-secreting cells were enumerated using an ELISPOT assay. Mice receiving EPI had significantly higher IgG antibody titers in serum than mice receiving s.c. immunization with the same vaccine formulation (Fig. 4A). Covering the target sites after EPI did not result in any changes in the IgG antibody level in serum. Both groups of mice receiving EPI had antigen-specific IgA spot-forming cells in their lamina propria. This is in contrast to the lack of IgA-positive cells in the lamina propria of s.c-immunized mice (Fig. 4B). There were no antigen-specific IgG spot-forming cells in the lamina propria with either EPI or s.c. administration (Fig. 4B). These data indicated that mucosal immune responses to EPI were not due to oral uptake of antigen administered to the skin with EPI.

**Cytokine responses.** IFN-γ and IL-4 responses were determined using spleen cells from the mice described for Fig. 4. EPI with PR8 virus plus CT and CpG DNA elicited a high level of IFN-γ (Fig. 5A) but no detectable IL-4 (Fig. 5B) responses in the ELISPOT assay. The IFN-γ response following EPI was consistently higher than that elicited by s.c. immunization with the same vaccine and adjuvants. The s.c.-immunized mice had a consistent IL-4 response. These data indicated that EPI elicited an immune response that had a Th1-biased cytokine profile.

**Protection against lethal challenge.** We have previously shown that EPI with a trivalent influenza vaccine (1998–1999) protected mice from a lethal challenge with a mouse-adapted A/Aichi influenza virus that was isolated 30 years earlier (9). No protection was observed if the vaccine was administered by s.c. injection (9). In the present study, challenge experiments were performed to determine the protective component of the trivalent vaccine and the effect of adjuvants on protection.

In the first study, mice (n = 8 per group) were immunized via the epidermal route on days 0 and 28 with the trivalent split vaccine or its monovalent components: A/Sydney, A/Beijing, and B/Harbin. Each animal received 1.5 µg of HA of the monovalent vaccine or a total of 4.5 µg of HA of the trivalent vaccine. Serum IgG antibody responses to the respective viruses were detected from blood collected 14 days postboost using ELISA (Fig. 6A). The antibody titers for all groups were in the range of 10^5 to 10^6. Mice were then challenged with 10 LD₅₀ of the A/Aichi virus 18 days postboost. All mice receiving the trivalent vaccine and seven out of eight mice immunized with the monovalent A/Sydney virus survived (Fig. 6B). How-
FIG. 2. IgG and IgA antibodies to PR8 virus in mucosal wash fluids. The mice described in the legend for Fig. 1 were sacrificed on days 42 and 49 (four mice/group/day) and mucosal samples were collected. IgG (A, C, and E) and IgA (B, D, F, and G) antibodies in the mucosal wash fluids from the small intestines (A and B), saliva (C and D), vagina (E and F), and nasal tract (G) were measured using an ELISA. Each data point represents an average OD value at 450 nm ± the standard deviation from two pooled samples (four mice each). The dilutions of the samples are shown in the figure.
ever, transient weight loss, up to 20% at days 4 to 6 postchall-
enge, was observed in all the surviving animals (data not shown). All naïve mice and mice immunized with A/Beijing and B/Harbin viruses succumbed to the challenge. These results indicated that protection against A/Aichi virus afforded by the trivalent vaccine was from the A/Sydney virus.

Antibody titers in the prechallenge sera were measured against the A/Aichi virus by ELISA. Cross-reacting antibodies were seen in all sera and the titers were comparable (Fig. 6C). The titers to the A/Aichi virus were approximately 1 log lower than titers to the immunizing virus. No hemagglutination antibody titers to A/Aichi virus were detectable in these sera at a 1:10 serum dilution (data not shown). This suggested that cross-reacting ELISA antibodies raised by the A/Sydney virus were responsible for the protection against challenge with the A/Aichi virus.

In the study evaluating the effect of adjuvant on protection, a prime-only immunization regimen was used because serum antibodies elicited by EPI with antigen alone by a prime-boost regimen could confer protection as shown above. Further, the differences in antibody levels in serum elicited by a prime-
boost regimen would make it impossible to differentiate the effect of adjuvant formulation over the nonadjuvanted formulation.

FIG. 3. IgG and IgA antibodies to PR8 virus in supernatants of mucosal fragment cultures. Tracheas and small intestines (SI) were collected from the mice described in the legend for Fig. 1 on days 42 and 49 and were used in the fragment culture assay. IgG (A) and IgA (B) antibodies in the culture supernatant (1:1) were determined in an ELISA. Each data point represents an average optical density value at 450 nm ± the standard deviation from two pooled samples (n = 4).

FIG. 4. Antigen-specific IgA antibody spot-forming cells in the mucosal tissues following EPI. Mice (n = 8 per group) were vaccinated on days 0 and 28 via EPI or s.c. routes with PR8 virus formulated with CpG DNA and CT. IgG titers in serum (A) were determined by ELISA; IgG and IgA (B) antibody spot-forming cells (SFC) in lamina propria of the small intestines were determined using an ELISPOT assay. The vaccination sites were covered in one group of mice receiving EPI for 24 h to prevent oral antigen consumption.
Mice were immunized by EPI with one of the following powder formulations: 1.5 μg of HA of A/Sydney virus alone, the same virus with 10 μg of CT, 10 μg of CpG DNA, or the virus with a combination of 10 μg of CT and 10 μg of CpG DNA. Antibody responses to the immunizing A/Sydney virus were measured in an ELISA assay with sera collected at 28 days postimmunization. All powder formulations elicited IgG titers in serum to the immunizing virus. The antibody titers were not statistically different between groups, although the combination adjuvant group appeared to have a marginally higher mean titer than the other two groups (P = 0.05) (Fig. 7A). The cross-reacting antibody to the A/Aichi virus was detected in all immunized mice, but the level was much lower than titers to the immunizing virus (Fig. 7A).

Mice were then challenged with 10 LD50 of a mouse-adapted A/Aichi virus at 32 days postvaccination. All eight naïve mice died within 8 days postchallenge. Five out of eight mice immunized with A/Sydney virus alone died, and the other three survived the 20-day monitoring period (Fig. 7B). The surviving animals had a 25% weight loss between 5 and 10 days after challenge and recovered 90% of the weight by day 20 (data not shown). The mice that were vaccinated with powdered vaccine containing CT or CT and CpG DNA all survived (Fig. 7B). These animals also experienced approximately 20% weight loss; the body weight recovery began on day 8, and complete recovery was seen for both groups on day 14 (data not shown). EPI with A/Sydney virus and CpG DNA resulted in protection in five of eight mice (62.5%). It was evident that the adjuvant formulations conferred superior protection over the adjuvant-free formulation, in spite of similar antibody levels in serum.

Because of this, antigenic challenge via any given route leads to different levels of immune response in different tissues. It is widely believed that the mucosal immune system can only be activated by direct antigenic challenge at a mucosal surface, whereas current immunization methods, e.g., i.m. injection, induce only a serum antibody response but not a mucosal antibody response (22, 24, 28, 34).

Previous studies indicate that some unique molecules, including CT and vitamin D3, may induce mucosal antibody responses when delivered via nonmucosal routes (13, 14, 16, 17). In certain cases, mucosal antibody responses could only be demonstrated to the adjuvant itself, but not to a coadministered antigen (16, 17). Nevertheless, these studies suggest that communication pathways may exist among various compartments of the immune system. In this study, we demonstrated that EPI with influenza viruses elicited a serum antibody response and a mucosal immune response that could be significantly enhanced by co-administration with adjuvants CT and CpG DNA. The IgA antibodies were detected in saliva and mucosal lavages from the small intestine, nasal mucosa, and the vaginal tract. Detection of sIgA antibodies released from the in vitro-cultured mucosal tissue fragments indicated the presence of IgA-secreting cells in the mucosal tissue. Identification and quantification of the frequency of IgA-secreting cells in the lamina propria provided further evidence of a local mucosal immune response that was elicited by EPI. The lack of detectable IgA antibodies in the sera from these mice confirmed the local origin of IgA antibodies. Our study suggests that skin may be an inductive site for mucosal immunity.

The structural and functional similarities between skin and mucosa may offer some clues with regard to the skin being an inductive site for mucosal immunity. Skin and mucosa are anatomically linked both at embryonic developmental stages and after birth. Functionally, skin and mucosa play a similar role as barriers to keep pathogens from entering the body. Furthermore, skin and mucosa are found to be effective induction sites for immune responses. Both skin and mucosa contain

**DISCUSSION**

Although compartmentalization of the immune system allows the host to focus its defense mechanisms to sites of infection, at the same time it presents an obstacle for vaccination strategies that aim to induce disseminated immune responses.
cells with active innate and adaptive immune functions. Finally, Langerhans cells in the epidermis and intraepithelial dendritic cells in the mucosa have common phenotypic properties and are potent antigen-presenting cells (2, 39, 41). One recent study revealed that dendritic cells from the skin, when activated by intradermal immunization, could migrate to the mucosal tissues (13). This is similar to the observation that immunization of one mucosal site leads to the migration of activated lymphocytes into diverse mucosal tissues (27, 30). The mechanism by which the mucosal immune response is induced by EPI requires further investigation.

The levels of influenza virus-specific IgG antibody observed in the mucosal lavages and fragment culture assays were very significant. This study did not determine what proportion of these antibodies were from local production as opposed to serum transudation. Numerous studies have indicated that the local production of IgG is an important component of the mucosal immune responses following traditional mucosal immunization or infection (1, 36, 37, 44). A recent study showed that passive transfer of serum antibodies to mice did not lead to appreciable levels of IgG transudation in fecal extracts and vaginal wash fluids (14). This evidence suggests that the influenza virus-specific IgG antibodies in the mucosal fluid observed in the present study may be from local production.

Irrespective of their origin, the mucosal IgG antibodies may be as important as the sIgA antibodies on the mucosal surface in providing protection against influenza virus. In the challenge experiment, we observed complete protection against mortality.

FIG. 6. Protection against a lethal challenge after EPI with adjuvant-free formulation. Mice (n = 8 per group) were immunized on days 0 and 28 with a trivalent (triva) or monovalent vaccine as follows: A/Sydney (A/Syd), A/Beijing (A/Beij), and/or B/Harbin (B/Harb). Mice were challenged on day 46 with 10 LD$_{50}$ of A/Aichi virus. Prechallenge (day 42) IgG titers in serum to each vaccine antigen (A), the percentage of survival (B) for up to 20 days postchallenge, and cross-reacting antibodies to A/Aichi virus (C) are shown.
of mice that were immunized with powder influenza vaccine and CT or CT and CpG DNA adjuvants. This is in contrast to the partial protection of the mice that were vaccinated with influenza vaccine without an adjuvant. The difference in protection may reflect the combined effect of IgG and IgA antibodies in the respiratory tract at the time of challenge since the antibody titers in serum were not different between the adjuvanted and nonadjuvanted groups.

The mechanisms by which the mucosal antibodies provide protection against an influenza virus that was isolated approximately 30 years ago remains to be elucidated. The current vaccine administered by i.m. injection offers protection by eliciting antibodies to the HA of the virus that can neutralize the infectivity of the virus. In the present study, antibodies to A/Sydney virus were found to have no HI titer to the Aichi/68 virus (data not shown). This is consistent with previous findings that HA offers little or no protection against viruses isolated 10 years apart (40, 42). Therefore, it appears that protection at the mucosal level was mediated by mechanisms other than HI antibodies. We speculate that non-HI antibodies (IgG and IgA) to the surface components of the virus may trap the virus in the mucus and prevent viral attachment and invasion.

Cytokines secreted by T-helper or other cells are critical in regulating the mucosal immune response. Some cytokines induce class switching in B cells, causing them to become IgA-producing cells. Other cytokines may induce IgA synthesis (28). Mucosal immunizations of mice often induce strong Th2-type cytokines. IL-4 and IL-5, in both the mucosal-associated lymphoid tissues and the spleen (45, 46). However, infection of mice with the influenza virus induces Th1-dominant cytokines in nasal-associated lymphoid tissue (25). Both the infection and the mucosal immunization were shown to induce mucosal IgA responses in these studies. Our preliminary data indicated that EPI generates a dominant Th1 type of cytokine profile. This cytokine profile mimics that induced by infection but is in sharp contrast to that from s.c. immunization. The distinct cytokine profiles in mice receiving EPI and s.c. immunization provided further evidence that different cell populations and regulation pathways are involved in immune responses to antigens administered via these routes. Further analysis of cytokine responses in the mucosal lymphoid tissues in future studies will help to delineate the role of cytokines in the mucosal immune response to EPI.

Our data suggest that the skin may be an inductive site for mucosal immune responses. However, induction of mucosal immunity appears to be dependent on the methods of immunization and the use of adjuvants. Applying CT or related molecules to the skin surface has resulted in the production of mucosal antibodies (18). Co-administration of 1,25(OH)2D3 with an antigen by intradermal injection induced mucosal antibodies to the vaccine (13). To our knowledge, there are no reports of induction of mucosal antibody responses when antigen alone or alum-adsorbed antigen was injected intradermally. Likewise, there were no reports of a mucosal response when liquid vaccine was injected using a jet-injector (35). It should be pointed out that a liquid jet-injector delivers vaccine largely to the subcutaneous tissue, although a trace amount of vaccine may be left on the entry track in the skin (35). Histological examination of the target site following EPI shows that powdered vaccines are deposited exclusively in the epidermis or the epidermal-dermal junction (8). The present study suggests that EPI with an appropriately formulated influenza vaccine offers a potential efficacy advantage over both i.m. injection and direct mucosal immunizations by eliciting augmented antibody responses in the serum and at the mucosal surface.

**ACKNOWLEDGMENTS**

We thank Ralph Brown and Scott Umlauf for evaluating the manuscript and for helpful discussions.
REFERENCES


7965

EPIDERMAL POWDER IMMUNIZATION

VOL. 75, 2001